Energy-Linked Pyridine Nucleotide Transhydrogenase Activity in Photosynthetically Grown *Rhodopseudomonas palustris*

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(Z. Naturforsch. **30 c**, 771–776 [1975]; received August 11, 1975)

Photosynthesis, *Rhodopseudomonas palustris*, Transhydrogenase, ATP, Energy Coupling

Rhodopseudomonas palustris (ATCC 17001) develops energy-dependent $NADP^+$ transhydrogenase activity while growing photosynthetically on thiosulfate, formate, or acetate as the electron donors. The enzymatic activity is present in the supernatant fraction S-144 000. — As reported, this fraction contains small membrane fragments but no closed vesicles and was shown to drive energy-dependent reversed electron flow as well as an aerobic respiratory electron transport. The energy-dependent transhydrogenase reaction in this fraction can be driven either by ATP, ADP, or inorganic pyrophosphate, but also by acetyl phosphate or acetyl-coenzyme A in the presence of orthophosphate. — Arsenate acts as an inhibitor and decreases preferentially the acetyl-coenzyme A-dependent and the acetyl phosphate-driven reaction; whereas, oligomycin inhibits preferentially the ATP- and the acetyl phosphate-dependent reactions. — Acetate kinase and a phosphotransacetylase are operative in S-144 000.

Introduction

The photosynthetic purple bacterium *Rhodopseudomonas palustris* (ATCC 17001, neo type 1974) is used for the preparation of the supernatant cell-free fraction S-144 000 after growth on thiosulfate as the electron donor [1, 2]. As reported, this fraction appears not to contain closed membrane vesicles [2–4]. The fraction, however, catalyzes a thiosulfate-linked, energy-dependent reversed electron transport in anaerobiosis to form reduced pyridine nucleotide. Energy is provided by energy-rich phosphates such as ATP, ADP, and PP_i [1, 2]. Moreover, under aerobic conditions, this cell-free system mediates the oxidation of reduced pyridine nucleotide [2]. The downhill electron flow responds to energy-rich phosphates, *i. e.*, it is retarded by ATP, by ADP, or by PP_i [2, 5]. This is assumed to be due to the ATPase system present in S-144 000 which can use as its substrate the energy-rich compounds, *i. e.*, ATP, ADP, and PP_i [2].

Conversely, the rate of the ATPase activity is decreased when reduced or oxidized NAD is added to S-144 000 [2]. Because this effect would decrease the ATPase coupled reverse electron flow, the possible existence of a $NADP^+$ transhydrogenase was examined. In this paper, evidence is presented for $NADP^+$ transhydrogenase activity in the supernatant fraction. It is shown that $NADP^+$ or NADPH does not decrease the ATPase activity and it is proposed that the NADPH rather than the NADH compound probably represents a reservoir for the reducing power generated in *Rps. palustris*. Furthermore, other energy-rich compounds besides ATP were tried as substitutes for ATP.

Abbreviations: A, absorbancy; AcCoA, acetyl-coenzyme A; AcP, acetyl phosphate; As_i, arsenate; CCCP, carbonyl-cyanide-*m*-chlorophenylhydrazone; DCCD, N,N′-dicyclohexylcarbodiimide; P_i, orthophosphate; PP_i, inorganic pyrophosphate.

Materials and Methods

Growth conditions of *Rps. palustris* and the preparation of the cell-free fraction S-144 000 were similar to those described earlier [2].

The oxygen uptake was measured polarographically. Reduction of pyridine nucleotide was followed spectrophotometrically by recording the ATP difference absorption changes at 340 nm in the Perkin-Elmer 356 two-wavelength double beam spectrophotometer. Much precaution was taken to follow the initial fast occurring rate of the pyridine nucleotide transhydrogenase reaction by using the proper relative concentrations of enzyme protein, pyridine nucleotides, and energy-rich substrates. The time difference which occurs between when the substrate is added and the recorder pen starts to follow the reaction is measured in sec. Where indicated, the traces of the reactions were extrapolated to zero time.

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The transhydrogenase activity was determined by following the reduction of $NADP^+$ under anaerobic conditions. A constant level of NADH was provided in both the sample and the reference cuvette by the addition of ethanol and alcohol dehydrogenase according to the method of Asano *et al.* [6]. The ATPase activity was determined as described previously [2].

Protein was measured by use of the biuret method of Gronall *et al.* [7].

Biochemicals were obtained from Boehringer Corp., D-6800 Mannheim. Oligomycin was delivered by Serva Corp., D-6900 Heidelberg.

More detailed information is given in the legends to the figures and tables.

Results

Transhydrogenase activity in Rps. palustris dependent on growth conditions

The presence of an active transhydrogenase system was tested in *Rps. palustris* grown under different conditions. The results are summarized in Table I. It could be shown that a transhydrogenase system is activated only under photosynthetic growth conditions when thiosulfate, formate, or acetate served as the electron donors. However, photoheterotrophic growth on malate or succinate depressed the transhydrogenase activity. Similarly, the growth in the dark with the electron donors malate or succinate as the energy- and carbon-sources and with oxygen as the electron acceptor does not lead to the development of an active transhydrogenase.

Table I. Transhydrogenase activity by S-144 000 from *Rps. palustris* after different growth conditions. The organism was grown either anaerobically in magnetically stirred 10-liter carboys maintained at ca. 35 °C in the medium given earlier [1]. Illumination was provided by 60 watt incandescent lamps providing 10 000 lx at the culture surface. Also, the cells were cultured aerobically at 35 °C in an 8 liter fermenter, Braun, Melsungen. The preparation of the cell-free extract for measuring transhydrogenase activity was described earlier [1].

Growth conditions	Electron donor	Transhydrogenase activity
anaerobically in the light	thiosulfate	+
„	formate	+
„	acetate	+
„	malate	−
„	succinate	−
aerobically in the dark	malate	−
„	succinate	−

Additional results were obtained with the supernatant fraction S-144 000 from the bacterium grown photosynthetically on thiosulfate.

Aerobic oxidation of NADH and NADPH by S-144 000, the retardation effect by energy-rich phosphates, and ATPase activity and the effect of NAD and NADP

As Fig. 1 indicates, S-144 000 is able to oxidize NADH faster than the NADPH. As reported [2, 5], the aerobic oxidation of NADH is retarted by energy-rich phosphates. Also, the oxidation of NADPH is retarted by ATP, ADP, or PP_i.

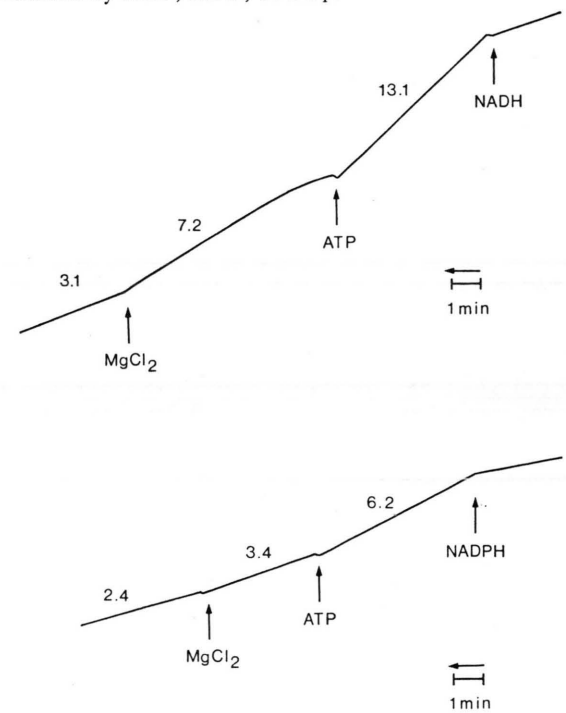

Fig. 1. Aerobic oxidation of NADH and NADPH by the supernatant fraction S-144 000. The reaction mixture in a total volume of 3.0 ml contained 95 μmol Tris-HCl (pH 8.0). 1 ml of S-144 000 was inserted which contained 14.5 mg protein. Where indicated by arrows, 5 μmol ATP and 2 μmol $MgCl_2$ were injected. The numbers indicate the amount of nmol oxygen taken up per 1 mg protein and 1 min. The oxygen uptake traces from the upper right to the lower left of the chart.

As the NADH oxidation is inhibited by ATP, the ATPase reaction rate started by the addition of 2 μmol ATP is decreased by 60% with an equal molar concentration of NAD^+ or NADH (Table II). $NADP^+$ or NADPH, however, does not cause this

Concentrations [μmol]						nmol P_i formed/ mg protein×min	%
ATP	$MgCl_2$	NAD^+	NADH	$NADP^+$	NADPH		
2	20	—	—	—	—	11.6	100
2	20	2	—	—	—	7.1	61
2	20	4	—	—	—	7.5	65
2	20	—	2	—	—	7.0	60
2	20	—	4	—	—	7.2	62
2	20	—	—	2	—	10.4	90
2	20	—	—	4	—	11.1	96
2	20	—	—	—	2	11.7	101
2	20	—	—	—	4	11.7	101

Table II. ATPase activity in S-144 000. The effect of NAD and NADP. The ATPase activity was determined as described previously [2]. The reaction mixture in a total volume of 2.8 ml was buffered with Tris-HCl, pH 8.0, 0.05 M, and contained the compounds listed in the table to the left 8.5 mg of S-144 000 cell-free protein were applied per reaction mixture. The numbers for nmol P_i released have been corrected for the endogenous amount of inorganic phosphate present. Additional control experiments were performed to determine the amount of inorganic phosphate detached non-enzymatically from the $NADP^+$ and NADPH molecules.

pronounced inhibition effect on the ATPase turnover (Table II).

These observations led to the assumption that an active $NADP^+$ transhydrogenase is operative in S-144 000.

The energy-dependent $NADP^+$ transhydrogenase reaction

Actually, the existence of an active ATP-dependent $NADP^+$ transhydrogenase can be observed in S-144 000. As Fig. 2 indicates, this reaction can be driven either by ATP, by ADP, or by PP_i (Fig. 2).

Under these conditions used, about 0.3 to 0.5 μmol $NADP^+$ are reduced per 1 mg protein and 1 min. This is true only for the first minutes following the start of the reaction (Table III). The stoichiometry of NADP reduced per ATP is in the order of 1.5 (Table III). Optimal conditions were obtained at pH 8.0. Equimolar concentrations of $NADP^+$ and ATP are sufficient to guarantee an optimal reaction rate.

As Table III indicates, to obtain energy-dependent transhydrogenase reaction in S-144 000, the ATP may be replaced by other energy-rich compounds such as acetyl phosphate and acetyl-coenzyme A in the presence of orthophosphate. Both the compounds acetyl phosphate and acetyl-coenzyme A are effective to the same extent as the ATP. The stoichiometry of these two reactions is given in Table III. A ratio of 1.2 to 1.5 is obtained for the amount of $NADP^+$ reduced per ATP or acetyl phos-

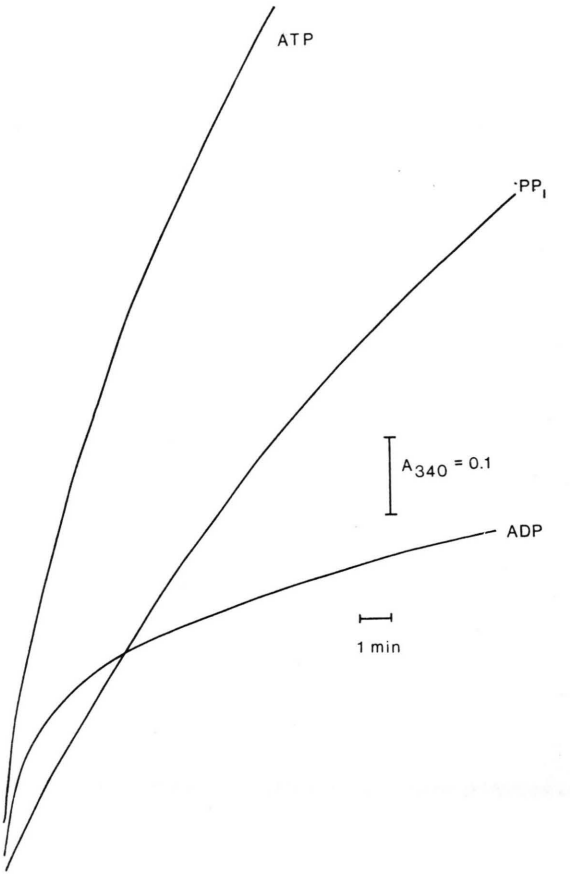

Fig. 2. Energy-dependent transhydrogenase reactions catalyzed by S-144 000. The reactions were carried out in anaerobic Thunberg-type cuvettes of 1 cm light path (see Materials and Methods). The main compartment of both the sample and the reference cuvette contained the reaction mixture in a total volume of 2.0 ml. The reaction mixture consisted of 56 μmol Tris-HCl (pH 8.0), 0.6 ml of S-144 000 containing 9.6 mg protein, 20 μmol $MgCl_2$, 30 μmol ethanol, 0.6 mg of yeast alcohol dehydrogenase (grade II, Boehringer, Mannheim), 0.4 μmol of both NADH and $NADP^+$. In addition, the side arm of the sample cuvette contained 5 μmol of either ATP, PP_i, or ADP. After evacuating the cuvettes, the energy-rich phosphates were mixed with the contents of the sample cuvette to start the transhydrogenase reaction. The reaction temperature was 25 °C.

Expt.	min	μmol inserted ATP	AcP	AcCoA +P_i	μmol $NADP^+$ reduced	Ratio $NADP^+$/ ATP; AcP; AcCoA +P_i	ATPase activity: μmol P_i liberated
1	0.8	0.2	—	—	0.30	1.5	0.034
2	2.9	0.4	—	—	0.55	1.4	0.029
3	2.5	0.6	—	—	0.75	1.25	0.093
4	0.4	—	0.2	—	0.32	1.5	—
5	2.2	—	0.4	—	0.50	1.25	—
6	2.1	—	0.6	—	0.80	1.3	—
7	0.9	—	—	0.2	0.18	0.9	—
8	1.0	—	—	0.4	0.33	0.8	—
9	1.0	—	—	0.6	0.47	0.8	—

Table III. Ratio of $NADP^+$ reduced and ATP, acetyl phosphate, and acetyl-coenzyme A plus P_i inserted in the transhydrogenase reaction. The reaction conditions were similar to those described under Fig. 3. The reaction mixture contained 1.0 ml of the 144 000 × *g* supernatant representing 10 mg of protein. The numbers for the μmol $NADP^+$ reduced have been calculated by extrapolating the traces of the reactions to zero time.

phate inserted. In case acetyl-coenzyme A plus orthophosphate serve as the energy source, the ratio of $NADP^+$ reduced and acetyl-coenzyme A applied is lower and was found several times to be in the range of 0.8 to 1.0.

Effect of inhibitors and uncouplers on the energy-dependent transhydrogenase reaction

Electron transport inhibitors, such as rotenone, antimycin A, and cyanide, at concentrations which will decrease, *e. g.*, the aerobic oxidation of NADH in S-144 000, are practically ineffective as far as the transhydrogenase activity is concerned. Likewise, uncouplers such as CCCP, even in 0.2 mM concentrations, do not reveal a distinct effect. Ionophorous antibiotics, *e. g.*, valinomycin, nigericin, and dianemycin also were tested and found to be without influence on the energy-dependent reaction when applied either alone or in the combinations valinomycin plus nigericin and valinomycin plus dianemycin (data not given).

However, arsenate inhibits the energy-dependent reaction. A concentration of 4 mM arsenate clearly decreased the ATP-driven reaction rate and a final concentration of 8 mM arsenate resulted in complete inhibition (Fig. 3: I). In case acetyl phosphate or acetyl-coenzyme A was used as the energy donor, a final concentration of 4 mM arsenate was sufficient to cause a complete inhibition of the transhydrogenase reaction (Fig. 3: II and III).

Oligomycin likewise causes a decreased transhydrogenase activity. However, the concentrations needed to cause an inhibitory effect were considerably higher compared to concentrations that are effective in mitochondria preparations [10] (Fig. 4).

The amount of 25 μg oligomycin per mg protein causes a definite inhibition rate when ATP or acetyl phosphate are taken as the energy donors (compare Fig. 4 to Fig. 3). A similar concentration of oligomycin, however, seems to be without effect on the acetyl-coenzyme A-driven reaction (Fig. 4). Doubling the concentrations of the antibiotic also results in a decreased transhydrogenase turnover (Fig. 4).

The transhydrogenase reaction is decreased also by dicyclohexylcarbodiimide (DCCD) at a final concentration of 8 mM. This is the case for the ATP-, acetyl phosphate-, and acetyl-coenzyme A-dependent reaction (data not shown).

Discussion

The ATP-dependent reversal of electron transfer by S-144 000 from *Rps. palustris* which results in the reduction of NAD^+ [1] was not understood completely in that the NAD^+ or NADH molecule decreases the ATPase turnover rate [2]. The proof of an active $NADP^+$ transhydrogenase in *Rps. palustris* supports the understanding of that step. The reduced NADH molecule which is generated through the ATPase-dependent reversed electron transport seems to be transhydrogenized immediately to yield the NADPH compound. In contrast to NADH, the NADPH molecule is protected better by a less active oxidase (Fig. 1) and, moreover, does not inhibit the ATPase reaction rate (Table II). The $NADP^+$ transhydrogenase reaction occurs at a rate which is 10 times faster than the ATPase turnover (Table III). It seems plausible that the $NADP^+$ transhydrogenase causes a pulling power on the ATPase-linked reverse electron flow. During photo-

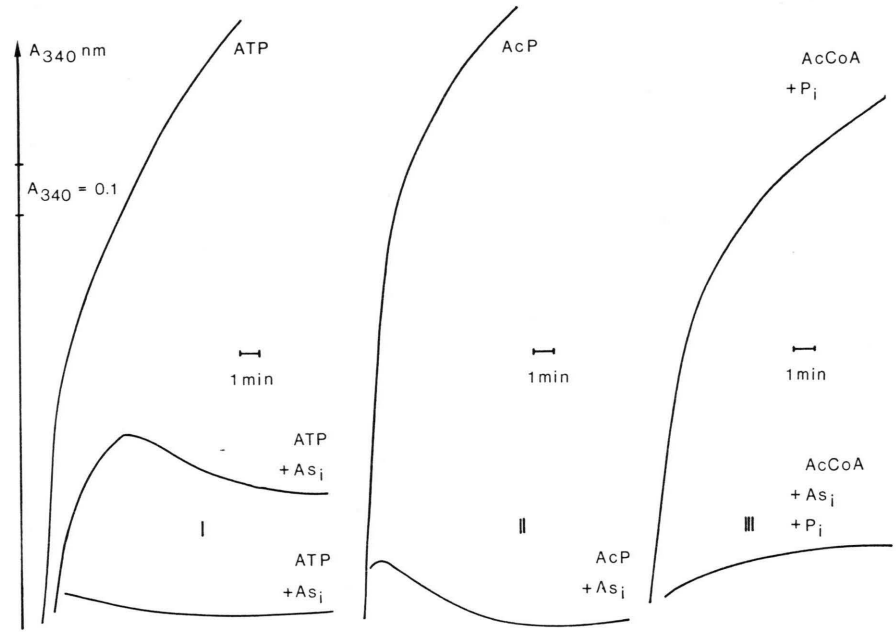

Fig. 3. Substitution of ATP by acetyl phosphate or acetylcoenzyme A (plus orthophosphate) as the energy sources for the transhydrogenase reaction in S-144 000. The experimental conditions were similar to those described in Fig. 2. In the different experiments, 6 μmol of ATP, or acetyl phosphate, or acetyl-coenzyme A (plus 6 μmol orthophosphate) were inserted. Acetyl-coenzyme A without an addition of orthophosphate does not reveal transhydrogenase activity. — I: Effect of arsenate on the ATP-dependent transhydrogenase reaction. The concentration of arsenate was 4 mM (upper curve) or 8 mM (lower curve), respectively. II. Action of arsenate on the acetyl phosphate-dependent reaction. The concentration of arsenate inserted was 4 mM. III: Influence of arsenate on the transhydrogenase reaction driven by acetyl-coenzyme A plus orthophosphate. Prior to the addition of orthophosphate (4 mM), arsenate (final concentration 4 mM) was added to the reaction mixture containing the cell-free extract. The amount of cell-free protein inserted was 12 mg per experiment.

synthetic growth of *Rps. palustris*, the energy-dependent reversal of electron transfer remains the only mechanism known to generate reducing power [11, 8, 9]. With substrates which would require photosynthetic growth conditions, an active transhydrogenase system is developed in the bacterium (Table I).

The $NADP^+$ transhydrogenase reaction was reported to operate also in particles from *Rps. spheroides* [13] and in membrane fragments of *R. rubrum* [14].

For *R. rubrum* chromatophore preparations, it was reported that during the ATP-dependent $NADP^+$ transhydrogenation approximately one ATP was consumed per one NADPH reduced [12]. This is in close agreement with the data obtained for the stoichiometry in the *Rps. palustris* cell-free extract S-144 000 (Table III).

It was found that the reaction in *Rps. spheroides* was driven either by light or by ATP in the darkness [13]. In the supernatant fraction S-144 000 from *Rps. palustris*, the ATP may be replaced by ADP (Fig. 2) which obviously is due to an active adenylate kinase present [2]. Likewise, inorganic pyrophosphate can serve as the energy donor in S-144 000 (Fig. 2). Chromatophore preparations from *R. rubrum* catalyze the same inorganic pyrophosphate-dependent $NADP^+$ transhydrogenation [14–16].

It is a new observation, however, that the energy-dependent transhydrogenation also functions in case acetyl phosphate or even acetyl-coenzyme A (in the presence of orthophosphate) functions as the energy donor (Fig. 3, Table III). Experiments to be performed on oxidative phosphorylation with the S-144 000 fraction as the enzyme source may help to clarify whether or not acetyl phosphate and acetyl-coenzyme A play a role in the phosphorylation or de-phosphorylation steps.

The effects of inhibitors and uncouplers on the S-144 000-mediated reaction (see Results) are as expected and consistent to those found in other systems [6, 17–21] (Figs 3, 4).

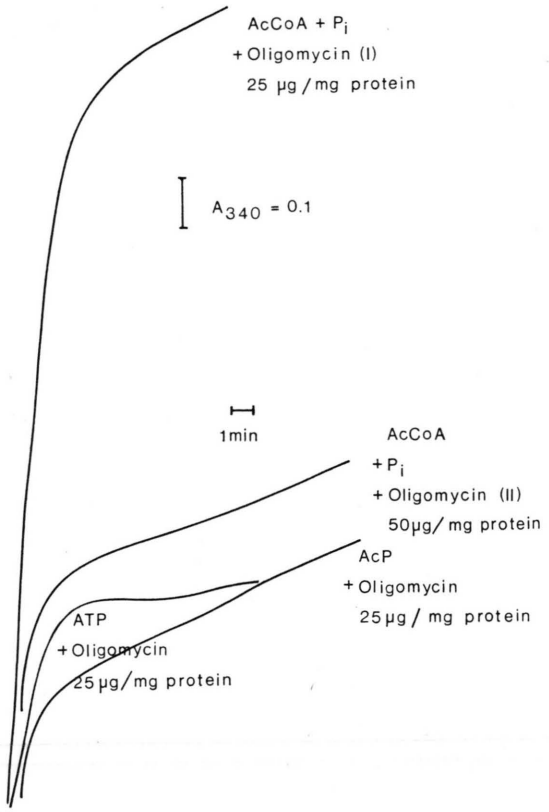

Fig. 4. Effect of oligomycin on the ATP-, acetyl phosphate-, and acetyl-coenzyme A-dependent transhydrogenase reaction: The reactions were performed as described in Fig. 2. Protein content was 14 mg per reaction. 0.4 µmol acetyl-coenzyme A (plus orthophosphate), acetyl phosphate, or ATP were added. The acetyl-coenzyme A-dependent reaction was started in the presence of 25 µg oligomycin per mg protein (I) and 50 µg oligomycin per mg protein (II), respectively. 25 µg oligomycin were inserted when the reaction was started by the addition of acetyl phosphate and ATP.

The presence of an active acetate kinase in S-144 000 can be demonstrated when the cell-free extract is incubated with acetate in the presence of ATP. The acetyl phosphate formed reacts with added coenzyme A and phosphotransacetylase (from *Clostridium kluyveri*) to form acetyl-coenzyme A and orthophosphate. Thus the increase of absorbancy at 233 nm due to the increasing concentration of acetyl-coenzyme A indicates the acetate kinase activity (data not given).

The effectiveness of acetyl-coenzyme A in the presence of orthophosphate to catalyze the energy-dependent transhydrogenase activity in S-144 000 indicates the active phosphotransacetylase present. The latter enzyme is known to be inhibited by arsenate (Fig. 3). It was reported that in many bacteria the acetate kinase activity was found constantly to parallel that of phosphotransacetylase [22].

Not only the energy-dependent $NADP^+$ transhydrogenase but also the energy-linked reversed electron transport in S-144 000 are operative when acetyl phosphate or acetyl-coenzyme A in the presence of orthophosphate serves as the energy donor (unpublished).

This work was supported by the Deutsche Forschungsgemeinschaft and partly by grants from the Friedrich-Alexander-Universität Erlangen-Nürnberg (No. I/6-130-10 and I/3-130-10). The skillful technical assistance of Herrn Klaus Pfleiderer is gratefully acknowledged. Thanks are due to Frau Ulrike Knoch-Dressel for preparing the figures.

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